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Effects of inhibition of phosphoinositide 3-kinase and p53 on monocytic differentiation driven leukemia cells with different p53 status

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Phosphoinositide 3-kinase (PI3-K) is an important mediator in promoting leukemia cell differentiation and survival signals. In contrast to previous studies, we have shown that inhibition of PI3-K by 2 h-pretreatment with the specific inhibitor LY294002 significantly increases p21 protein expression in phorbol 12- myristate 13-acetate – treated leukemia cell lines with different p53 status. This occurs within 2–6 h of treatment of human acute promyelocytic leukemia NB4 (p53-positive) and acute monocytic leukemia THP-1 (p53-negative) cells driven to monocytic differentiation. The up regulation of p21 expression correlates with the activation of targets of the PI3-K signaling pathway, such as extracellular signal regulated kinase (ERK) 2, protein kinase C ζ, transcription factor Sp1, and p21 transcriptional regulation by Sp1. LY294002 does not induce p21 expression in NB4 cells driven to granulocytic differentiation by retinoic acid or the histone deacetylase inhibitor phenyl butyrate. Piffithrin-α, an inhibitor of p53 transcriptional activity, does not influence the p21 expression level or changes the protein expression profile. Furthermore, piffithrin-α induces apoptosis, but differently influences leukemia cell viability, protecting only p53-positive leukemia cells from phenyl-butyrate – induced death. To conclude, this study provides evidence for relationship between PI3-K, ERK pathways and p53 in defining leukemia cell fate.

Key words: leukemia, PI3-kinase, PMA, piffithrin-α, nuclear proteins

INTRODUCTION

Phosphoinositide 3-kinase (PI3-K) is a class of enzymes that participates in a cellular processes linked to cell growth, differentiation, apoptosis and cytoskeleton rearrangement. One of the main functions of PI3-K is the phosphorylation of inositol-containing membrane lipids acting as intracellular mediators themselves and activating many effectors, such as protein kinases, phospholipase, G-proteins [1]. PI3-K activity is tightly regulated in normal cells by various mechanisms. Akt, known as protein kinase B (PKB), is a crucial downstream target of PI3-K [2]. It is known that Akt can affect proliferation through signals to the cell-cycle machinery allowing cyclin D1 accumulation, which is important for the G1/S phase transition [3]. The blockade of PI3-K or Akt activity leads to cell-cycle arrest. Akt can also negatively influence the expression of cyclin-dependent kinase (CDK) inhibitors, such as Kip1 (p27) or Cip1 (p21) [4, 5]. Akt can modulate activity of p21, which is a marker for growth arrest and differentiation, by affecting its phosphorylation and binding to proliferating cell nuclear antigen (PCNA) [6]. In some cancer cells, PI3-K is involved in the negative regulation of p21 activity; for instance, PI3-K inhibition by LY294002 potentiates zinc-induced expression of p21 in the colorectal cancer cell line HT29 [7]. The p21 expression is controlled at the transcriptional level by both p53-dependent and independent mechanisms and at the posttranscriptional level [8]. A variety of agents activate p21 by binding different transcription factors to specific *cis*-acting elements in the p21 promoter. For example, phorbol 12-myristate 13-acetate (PMA), ocadaic acid or histone deacetylase (HDAC) inhibitors stimulate p21 promoter in cancer cells through the involvement of the transcription factor Sp1 [9, 10].

PI3-K has been shown as a strong factor of survival blocking apoptosis. PI3-K activates Akt, which then modifies binding properties by phosphorylation of the pro-apoptotic Bcl-2 family member, BAD, resulting in cell survival [11]. The inhibition of PI3-K reduces cell survival, induces apoptosis or sensitizes leukemia cells to apoptosis in combination with cytotoxic drugs [12]. Akt

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can influence cell survival by indirect effects on two central regulators of cell death, i.e. the anti-apoptotic nuclear factor of κB (NFκ-B) and pro-apoptotic tumor suppressor, p53 [13–15]. Akt acts through phosphorylation of Mdm2 protein, which then translocates to the nucleus where it binds p53 and results in enhanced p53 degradation by the proteosome and thus in survival [14, 15].

The induction of erythroid or granulocytic differentiation has been shown to be associated with an increase in PI3-K activity [16, 17]. The specific inhibition of PI3-K activity prevents vitamin D3- or PMA-induced monocytic and retinoic acid (RA)-mediated granulocytic differentiation of promyelocytic leukemia HL-60 cells [17–19]. Members of the protein kinase C (PKC) family, which have a different cytoprotective influence, participate in mediating PI3-K signaling. It has been demonstrated that PI3-K activity is essential for the activation of downstream atypical protein kinase C, PKCζ, which leads to growth arrest, differentiation to monocytes/marcrophages and survival [18, 19]. The PI3-K pathway is important for growth regulation and apoptosis and often cross-talks with the extracellular kinase ERK pathway [20]. However, there is still little insight into how PI3-K regulates downstream targets in response to therapeutical drugs in diverse leukemia cell lines in association between p53 status and drug sensitivity.

In this study, we found unusual effects of PI3-K inhibition on PMA-induced responses of the leukemia cell lines NB4 and THP-1 with a different p53 status, and we have investigated the downstream targets – cellular proteins and transcription factors that may be involved in these effects.

MATERIALS AND METHODS

Cell cultures. The human leukemia cell lines (promyelocytic HL-60, acute promyelocytic NB4 and acute monocytic THP-1) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Grand Island, NY) at 37 °C in a humidified 5% CO_2 atmosphere and used for assays during the exponential phase of growth.

Cell viability and growth. Cell viability was assayed by exclusion of 0.2% trypan blue. Cell number was determined by counting cells in suspension in a haemocytometer.

Preparation of nuclear extracts. Adherent cells were gently scraped from plastic; those and nonadherent cells were harvested and pelleted at 500 × g for 6 min and washed twice in ice-cold PBS. Nuclei were prepared using the Nuclei Isolation Kit (Sigma) according to the manufacturer's recommendation. Nuclei were completely suspended in the Nuclei EZ storage buffer and frozen at –76 °C. Nuclear protein extracts for the electrophoretic mobility shift assay (EMSA) were prepared by lysis of nuclei in a buffer containing 20 mM Tris-HCl, pH 8.0, 200 mM EDTA, 2 mM EGTA, 20% glycerol, 400 mM NaCl and inhibitors: 1 mM phenylmethylsulfonylfluoride (PMSF), 3 mM dithiothreithol (DTT) and Complete protease inhibitor cocktail (Roche, Germany). After incubation for 1 h on ice, the extracts were centrifuged at 18,000 × g for 20 min and used immediately. Protein concentrations were measured using commercial RCDC Protein Assay (Bio Rad).

Electrophoretic mobility shift assay (EMSA). The probes used were synthetic oligonucleotides representing binding sites: (5'-GCCTGGGCCCCGGGAGGG GGTCCCGGGCGGCGC-3') Sp1 (elements 1 and 2) from the p21 promoter; (5'-ATTCGATCGGGGCGGGGCGAGC-3') consensus Sp1; (5'-ATCAGGAACATGT CCCAACATGTTGAGCTCT-3') p53 from the p21 promoter. Complementary oligonucleotides were annealed and labelled at their 5' ends using [γ-^{32}P-ATP] (Amersham, U.K.) and T4 polynucleotide kinase (Fermentas, Vilnius, Lithuania). Standard DNA reactions were performed with 15 μg nuclear extracts in a 20 μl of reaction buffer (10 mM HEPES pH 7.9, 3 mM $MgCl_2$, 0.1 mM EDTA, 40 mM NaCl, 10% glycerol) containing 2 μg bovine serum albumin (BSA), 1 μg poly(dI-dC), 1 pM labelled oligonucleotide for 30 min at room temperature. When desired, the unlabeled competitor oligonucleotide was added to protein extracts at a 100-fold molar excess for 15 min of preincubation. DNA–protein complexes were resolved on 6% polyacrylamide gel containing 1 × Tris-borate buffer. After electrophoresis, the gels were dried and then exposed to X-ray films.

Isolation and fractionation of total cell protein. Cells were resuspended (5×10^7 cells/ml) in 2 volumes (v/v) of 2 × lysis solution (100 mM Tris, pH 7.4, 5 mM $MgCl_2$, 200 mM DTT and 4% SDS), then three volumes of 1× lysis solution and benzonase (Pure Grade, Merck, Germany) to give 2.5 U/ml as a final concentration were added. The lysates were incubated for 1 h at 0 °C and then centrifuged at 15,000 × g for 30 min. The supernatants were immediately subjected to electrophoresis or saved at –76 °C.

Gel electrophoresis and immunoblot analysis. Total cell proteins were resolved by SDS electrophoresis (SDS/PAGE) using 6–18% polyacrylamide gradient gel (Invitrogen, CA) in a Tris-glycine electrophoresis buffer. After SDS electrophoresis, proteins were transferred to Immobilon™ PVDF membranes and blocked by incubating with 5% BSA dissolved in PBS containing 0.18% Tween-20 overnight at 4 °C. The membranes were incubated for 1 h with antibodies against proteins examined (Santa Cruz, Biotechnology, Inc., CA) at a concentration of 1 μg/ml in PBS containing 0.18% Tween-20, 0.35 M NaCl, and 1% BSA. The membranes were subsequently washed with PBS-Tween-20 and then incubated with a horseradish peroxidase-conjugated secondary antibody (DAKO, A/S, Denmark) for 1 h at room temperature. Thereafter, the filters were washed as described and immunoreactive bands detected by enhanced chemiluminescence using ECL™ Western blotting detection reagents (Amersham Pharmacia, Sweden) according to the instructions of the manufacturer.

RESULTS AND DISCUSSION

The p53 gene is one of those most frequently altered in human cancer. Previous studies have suggested that expression of p53 in cancer cells can result in different responses. To investigate the basis of these differences, we used NB4 cells harboring the p53 gene and p53-null THP-1 cells. Both cell lines were treated with 100 nM PMA, which induced differentiation into monocytes with a characteristic cell spreading and adhesion on plastic at 2–8 h. To test whether PI3-K could affect the cell fate, we used a specific inhibitor of PI3-K, LY294002 (30 μM), for 2 h before induction of differentiation by PMA. Cells pretreated with LY294002 cells and PMA-treated maintained cell adhesion during 12 h in culture. We first investigated the response of PI3-K activity interruption on the targets of this signaling pathway (p21, p53, p38, Sp1, PKCζ) and the involvement of certain transcription factors in these events.

One of the p21 transcription mechanisms involves the regulation by binding transcription factors to the p21 promoter. This promoter contains six binding sites for transcription factor Sp1 and two for p53. In order to determine whether the Sp1 is involved in p21 transcription, for EMSA we used oligonucleotides corresponding to the Sp1 consensus motif or to Sp1 binding sites 1 and 2 known as specific for Sp1-mediated p21 gene induction in response to PMA [21]. The formation of DNA–protein complexes was observed; their specificity was confirmed by a competition using an excess amount of unlabeled oligonucleodide (cold) which eliminated detection of complex formation. Using as a probe the Sp1 consensus motif, the intensity of bands was markedly increased when the nuclear extracts from PMA-treated NB4 cells were used (Fig. 1). The maximal binding intensity was noticed at 6 h of PMA-treatment. PI3-K inhibition by LY294002 did not change Sp1 binding activity to the consensus motif. However, Sp1 binding to specific sites 1 and 2 of the p21 promoter was enhanced at 6 h in NB4 or THP-1 cells treated with PMA or PhB, respectively, following pretreatment with LY294002. As shown in Fig. 2, pretreatment with LY294002 rapidly increased p21 protein level at 2 h with a remarkable enhancement at 6 h in both NB4 and THP-1 cells suggesting a p53-independent event. As can be seen from Western blot analysis, NB4 cells (but not THP-1) express p53 protein. However, both cell lines respond to PMA that induce cell differentiation to monocytes. This moment is essential, because in NB4 cells we did not detect any increase in p21 expression after LY294002 pretreatment before the induction of granulocytic differentiation by retinoic acid (RA) (data not shown). Also, we found a coordinated increase in Sp1 protein level at 2 h of indicated treatment in both cell lines. LY294002 and PMA, administered individually, did not show enforced p21 expression at 2 h of treatment in NB4 and THP-1 cells. The HDAC inhibitor PhB caused an increase in the expression of p21 at 6 h and no additional effect following pretreatment with LY294002 in NB4 cells (Fig. 2). Such treatment also enhanced PKCζ expression in THP-1 cells at 2 h and in NB4 cells at 6 h. Indeed, exposure of cells to HDAC inhibitors usually results in up regulation of p21, which inhibits cell cycle progression and may also exert anti-apoptotic actions, possibly by blocking the activation of caspases [22–24].

As shown in Fig. 3, combined treatment with LY/PMA in THP-1 cells caused a marked expression of the phospho-

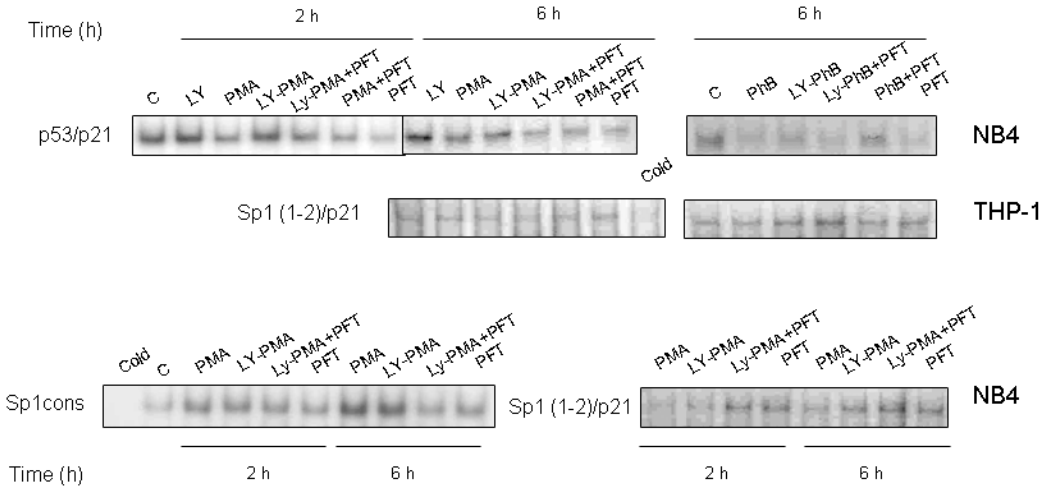

Fig. 1. Changes of transcription factors activity in response to PI3-K inhibition in leukemia cells treated with PMA and PhB in the absence or the presence of PFT

Nuclear extracts were prepared from NB4 and THP-1 cells treated with 30 μM PFT, 25 μM LY294002, 100 nM PMA and 4 mM PhB for 2–6 h or pretreated for 2 h with 25 mM LY294002 before exposure to PMA or PhB without or with 30 μM PFT for indicated time-points. EMSA was performed using a total 15 μg protein from each nuclear extract and oligonucleotides containing the Sp1 consensus motif, Sp1 or p53 binding sites from the p21 promoter. Specific DNA complexes with p53 or Sp1 were eliminated competitively by addition of a 100-fold molar excess of unlabeled oligonucleotide (cold).

rylated form of ERK2 at 2 h only and a subsequent reduction in the level of this phosphorylated/activated mitogen activated protein kinase (MAPK). In addition, exposure of the cells to LY294002 before PMA treatment resulted in an increase of p38 MAPK activation by phosphorylation at the same time-point as well.

In immature monocytes, p21 is expressed in the nucleus and induces G1 cell cycle arrest, which is necessary for the induction of differentiation [25, 26]. While the differentiation program to monocytes takes place, the nuclear localization of p21 becomes cytoplasmic [24]. Another important role for p21 is the protection of cells against apoptosis [27, 28]. The cytoplasmic p21 forms a complex with apoptosis signal-regulating kinase 1 (ASK1), over expression of which induces apoptotic cell death [29]. In the case of monocytes, such complex formation is directly responsible for resistance to apoptosis by inhibition of activation of the MAP kinase cascade which participates in the amplification of the apoptotic process [24]. Increasing evidence suggests that the PKC family of kinases is involved in the regulation of critical cell cycle transitions, which is dependent on the isoform involved [30]; for instance, PKCζ is activated through a PI3-K-dependent mechanism in protecting cells from apoptosis.

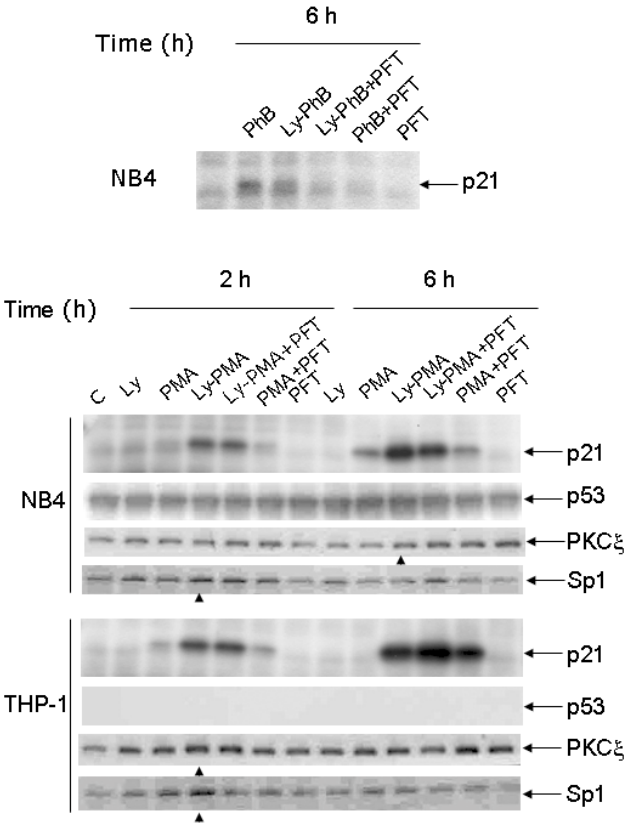

Fig. 2. Expression profiles of proteins in response to PI3-K inhibition in leukemia cells treated with PMA in the absence and the presence of PFT

NB4 and THP-1 cells were treated with 30 μM PFT, 25 μM LY294002 and 100 nM PMA for 2–6 h or pretreated for 2 h with 25 μM LY294002 before exposure to PMA or 4 mM PhB without or with 30 μM PFT for indicated time-points. Total proteins were fractionated on SDS-PAGE (6–18%) and analyzed by Western blotting with antibodies directed against each protein.

The relation between maturation and apoptosis is complex, and the differentiation program can itself trigger cell death [31]. We have shown previously that in leukemia cell lines, HL-60 and NB4, 100 nM PMA induced monocytic differentiation associated with the up regulation of p21 cytoplasmic expression, which was down regulated in apoptosis-undergoing cells [32]. In this context, PI3-K inhibitors, such as LY294002 and wortmannin, have been shown to block RA- and PMA-mediated maturation of HL-60 cells [33, 34]. The interruption of Akt signaling by LY294002 may disrupt cytoprotective signaling pathways, resulting in induction of apoptosis. In fact, our results, demonstrating an enforced p21 expression by LY294002/PMA, argue against the possibility that Akt down regulation is responsible for cell death potentiation. However, p21 also antagonizes the apoptosis that occurs by binding to and inhibiting caspase-3 [35]. In addition, the ability of rapid ERK2 activation by LY294002/PMA regiment may attenuate cell death too. PI3-K has a major role in coupling integrins to ERK2 activation in leukemia cell adhesion [36]. The observation that MAPK lies upstream of p21 points to the requirement of p21 for differentiation induction [24], although the bulk of evidence suggest that MAP kinases like ERK1/2 promote cell survival, whereas p38 facilitates cell death [37]. In our experimental model, exposure to LY294002/PMA temporally increased p38 expression which declined after 6 h of treatment. Thus, induction of p21 is dependent

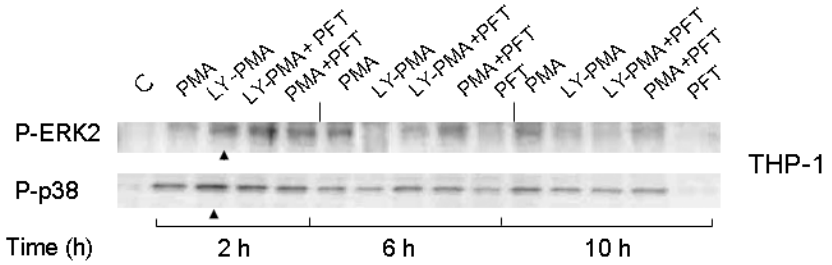

Fig. 3. Effects of interruption of PI3-K pathway on the activation of MAPK pathway proteins. THP-1 cells were treated with 30 μM PFT, 25 μM LY294002 and 100 nM PMA for 2–6 h or pretreated for 2 h with 25 μM LY294002 before exposure to PMA without or with 30 μM PFT for indicated time-points. Total proteins were fractionated on SDS-PAGE (6–18%) and analyzed by Western blotting with antibodies directed against phosphorylated forms of ERK2 and p38.

upon activation of the ERK pathway and differentially modulated by inhibition of PI3-K activity.

The physiological role of p53 is to maintain the integrity of the genome. In response to genotoxic stress, the elevation of p53 level permits repair processes or the initiation of apoptosis [38]. Thus, suppression of p53 function may be exploited for therapeutic advantage. Recently, a synthetic, water-soluble, and stable compound, piffithrin-α (PFT), has been reported to be a specific inhibitor of p53 transactivation and p53-dependent apoptosis therapy [39]. The exact mechanisms of PFT are not clear, because data on its effects on apoptosis are controversial. To clarify the action of PFT, we examined the effects of PFT itself and in combination with an apoptosis-inducing agent, PhB, on leukemia cells with a different status of p53 (NB4 and THP-1). The results (Fig. 4) demonstrated that long-term treatment with 30 μM PFT induced cell death in both cell lines, in contrast to the data [39] showing PFT as an inhibitor of p53-dependent apoptosis. Moreover, PFT-induced apoptosis was more prominent in p53-positive NB4 cells. A high dose (4 mM) of PhB induced apoptosis in both cell lines with different efficacy; THP-1 cell apoptosis was delayed and reached the same level on day 8, versus day 5 in NB4 cells (data not shown). Interestingly, a long-term co-treatment with PFT and PhB for 5 days rescued p53-positive NB4 cells from PhB-induced apoptosis, but enhanced cell death in p53-negative THP-1 cells. Thus, these observations indicate that PFT-induced apoptosis may be mediated through p53-dependent and p53-independent signaling pathways.

We tested the effect of PFT on p53-mediated events, i.e. p53 binding activity to the p21 promoter and p53 expression in NB4 cells treated with LY294002/PMA (Fig. 1). Exposure of NB4 cells to PFT alone or in combination with PMA with or without LY294002 pretreatment resulted in a decrease of p53 binding activity, but did not change the level of p53 or p21 protein expression (Fig. 2). However, PFT alone or in combination with PMA stimulated PKCζ expression at 6 h in NB4 and THP-1 cell lines.

The occurrence of p53-mediated apoptosis has been reported to be relevant to the activation of MAP kinases [40, 41]. Here, we tested whether PFT affects activation by phosphorylation of ERK and p38 kinases in p53-negative THP-1 cells. PFT alone or in combination with PMA or LY294002/PMA had no apparent effects on ERK2 and p38 phosphorylation. During the indicated experiments, apoptosis was not induced, suggesting that the changes in intracellular MAP kinase activities are not required for the action of PFT in p53-negative cells.

Overall, it looks like that PFT is not a specific inhibitor of p53-mediated signaling and apoptosis, at least in our experimental system, indicating that PFT may not specifically target p53, but targets some unknown cellular components of the major signal transduction pathways.

Taken together, our findings suggest that the signaling cascades that regulate the response to PI3-K interruption by affecting its downstream targets may vary with the cell type and/or the stimuli.

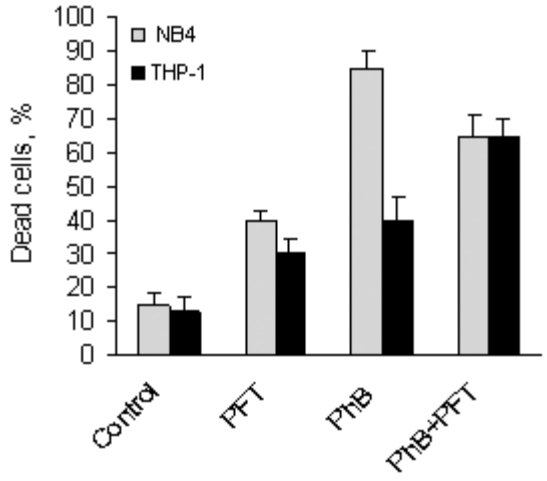

Fig. 4. Effects of PFT on survival of leukemia cells with different p53 status

NB4 and THP-1 cells were incubated with 30 μM PFT alone or in combination with 4 mM PhB. Aliquots of the cultures were subjected to counting on day 5 following staining with 0.2% trypan blue for the determination of the total number of dead cells. Results are mean ± SEM (n = 3).

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FOSFOINOZITIDO 3-KINAZĖS IR P53 INHIBAVIMO POVEIKIS INDUKUOTŲ LEUKEMINIŲ LĄSTELIŲ SU SKIRTINGU P53 STATUSU MONOCITINEI DIFERENCIACIJAI

Santrauka

Fosfoinozitido 3-kinazė (PI3-K) yra svarbus tarpininkas perduodant signalą vėžinių lastelių diferenciacijos ir išgyvenimo procesuose. Priešingai ankstesniems darbams, aptikta, kad PI3-kinazės aktyvumo blokavimas specifiniu inhibitoriumi LY294002 2 val. iki monocitinės diferenciacijos indukcijos su forbolio 12-myristato 13-acetatu labai padidina p21 baltymo ekspresiją žmogaus ūmios leukemijos ląstelėse. Efektas pasireiškia 2–6 val. tiek promielocitinės leukemijos NB4 ląstelėse, turinčiose p53 geną, tiek ir jo neturinčiose monocitinės leukemijos THP-1 ląstelėse, indukuotose monocitinės diferenciacijos. Šis efektas yra lydimas PI3-K signaliniame kelyje dalyvaujančių baltymų (ERK2, p21, p38, PKCζ, Sp1) ir p21 baltymo transkripcijos reguliatoriaus Sp1 aktyvinimo. NB4 ląstelėse, indukuotose granuliocitinės diferenciacijos su retinoine rūgštimi ar apoptozės su histonų deacetilazių inhibitoriumi, fenilo butiratu, LY294002 neturėjo įtakos p21 ir kitų tirtų baltymų ekspresijai. Pifitrinas-α, p53 transkripcinio aktyvumo inhibitorius, abiejose ląstelių linijose indukavo apoptozę; panaudotas kartu su fenilo butiratu pasižymėjo skirtingu poveikiu leukeminių ląstelių išgyvenimui ir apsaugojo nuo žūties tik ląsteles su p53 genu. Išvada: šio darbo rezultatai rodo PI3-K signalinio kelio ir su juo susijusių ląstelės baltymų bei transkripcijos veiksnių įtaką leukeminių ląstelių diferenciacijai ir žūčiai.